

# Alteration of Concanavalin A Binding Glycoproteins in Cerebrospinal Fluid and Serum of Alzheimer's Disease Patients

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Alzheimer's disease (AD) is the most common cause of dementia in the elderly. It is characterized pathologically by the formation of senile plaques and neurofibrillary tangles in the brain. Diagnostic markers for detecting earlier stages of AD are needed. We measured the intensity of concanavalin A (Con A) binding activities of glycoproteins of the cerebrospinal fluid (CSF) and serum of subjects to clarify the modification of core mannose since we expected that aberrant glycosylation of glycoproteins might be useful as a new biomarker for detecting AD. CSF samples were collected from 15 patients with probable AD (AD group), 5 patients with probable dementia with Lewy bodies (DLB) (DLB group) and 8 controls without dementia (control group), whereas serum samples from 20 patients with probable AD and 20 controls without dementia were also collected. Glycoproteins in the CSF and serum were detected by lectin blotting using Con A. In the CSF of the AD group, 2 Con A binding glycoproteins were significantly higher compared with the control group. Furthermore, using analysis of variance, 3 Con A binding glycoproteins detected from the CSF of the AD group showed significant differences among the 3 groups. The levels of 3 Con A binding glycoproteins were significantly lower than in non-dementia controls in the serum. These changes in Con A binding activities did not depend on the amount of proteins. Therefore, the data indicate that the aberrance of protein glycosylation relates to the pathology of AD, and has some promise as a new biomarker for the diagnosis of AD.

**Key words:** Alzheimer's disease; concanavalin A; glycoprotein; glycosylation

Alzheimer's disease (AD) is an age-related dementia characterised pathologically by the appearance of senile plaques composed primarily of aggregated forms of  $\beta$ -amyloid protein and neurofibrillary tangles in the brain (Glennner and Wong, 1984; Grundke-Iqbal et al., 1986). Since the 1990's, AD has become the most common cause of dementia in Japan (Urakami et al., 1998). AD is not only a medical care issue but also a social issue, because of the rapid aging of the population in Japan and other countries.

Recently, donepezil hydrochloride, an inhibitor of acetylcholinesterase, was created for the treatment of AD (Yamanishi et al., 1998). It is used as a treatment for mild to moderate AD patients in Japan (Homma et al., 2000), and its effectiveness in mild cognitive impairment, which is the prodromal stage of AD, has been reported (Salloway et al., 2004). Clinical diagnosis of AD is based on a clinical interview of the patient's medical history, neuropsychiatric examinations and neuroimaging studies. However, there is a matter

Abbreviations: AD, Alzheimer's disease; Con A, concanavalin A; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies

of objectivity in current diagnostic criteria for AD. Therefore, specific biological diagnostic markers for detecting earlier stage of AD and specific distinction of AD are becoming more important.

The need to improve diagnostic accuracy has prompted the search for diagnostic markers in the cerebrospinal fluid (CSF) or serum of persons with AD. To date, no single biochemical marker has been found to yield sufficient sensitivity and/or specificity of detection. Nevertheless, the combination of multiple biomarkers such as tau,  $\beta$ -amyloid 1-42 and -40 (Galasko et al., 1998; Kanai et al., 1998) may improve diagnostic accuracy. Additionally, there are new possible biomarkers for the diagnosis of AD such as apolipoprotein A-1, cathepsin D, hemopexin, transthyretin and 2 pigment epithelium-derived factor isoforms (Abdi et al., 2006; Castano et al., 2006). Identification of new biological markers, which can be used alone, or in combination with other markers is highly necessary.

Among the more than 100 types of post-translational modifications, glycosylation is the most common. N-type glycosylation on asparagines and O-type glycosylation on serine or threonine occur in over 50% of all proteins (Varki et al., 1999). Altered glycosylation is reported in several disease states (Lis and Sharon, 1986; Collinge et al., 1996) and in AD (Ahmed and Thornalley, 2002). Glycosylation of proteins with glucose and other saccharide derivatives leads to the formation of fructosamines and advanced glycation endproducts (Day et al., 1979; Furth, 1988). Formation of the endproducts has been linked to the development of AD (Dukic-Stefanovic et al., 2001). Additionally, neural sialyltransferase, which catalyses the addition of terminal sialic acid onto newly synthesized proteins following removal of core mannose sugars (Paulson and Colley, 1989; Harduin-Lepers et al., 1995), is decreased in the brains of AD patients (Maguire and Breen, 1995).

Lectins are powerful tools for distinguishing differences in the carbohydrate composition of glycoproteins (Lis and Sharon, 1986; Elgavish

and Shaanan, 1997). Concanavalin A (Con A) is one of the lectins that targets the core mannose (Ohyama et al., 1985). We examined the alteration of core mannose modification by measuring Con A binding activities of glycoproteins in the CSF and serum. In this study, we showed the difference in glycosylation between the CSF and serum, and the ability of a new biomarker of AD.

## Materials and Methods

### *Subjects and samples*

Before collecting samples, informed consent was obtained from the patients and their families, and ethical investigation was approved. All subjects were examined by a dementia specialist (KU). Then, interviews of medical history, neuropsychiatric examinations and neuroimaging studies, such as computed tomography and/or magnetic resonance imaging, single photon emission computed tomography were carried out for the diagnosis. AD diagnosis was done according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (American Psychiatric Association, 1994) and the National Institute of Neurological Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association Criteria (McKhann et al., 1984). Dementia with Lewy bodies (DLB) is characterized by persistent visual hallucinations, fluctuating cognitive impairment and parkinsonisms, and typical senile dementia. The diagnosis for DLB patients was done using the diagnosis in the consensus guidelines for the clinical and pathologic diagnosis of DLB (McKeith, 2006). Non-dementia control subjects had no cognitive impairments or diseases of the nervous system, yet may have had some other diseases not related to dementia. All samples were immediately frozen immediately after collecting until experimentation.

CSF samples were collected from 15 patients with probable AD, 5 patients with probable DLB and 8 non-dementia controls (Table 1). The AD group

consisted of 8 males and 7 females (mean age  $\pm$  SD,  $73.8 \pm 5.2$  years), the DLB group, 3 males and 2 females ( $77.2 \pm 3.4$  years) and the control group, 3 males and 5 females ( $73.3 \pm 8.5$  years).

Serum samples were collected from 20 probable AD patients and 20 non-dementia controls (Table 2). The AD group was made of 7 males and 13 females ( $79.2 \pm 6.8$  years), and the control group, 4 males and 16 females ( $71.6 \pm 12.0$  years). This study did not use the same subject sample between the CSF and serum.

### Pretreatment of samples

The quantity of total protein included in the CSF and serum were determined by absorptiometry using Protein Assay Kit II (Bio-Rad, Hercules, CA), and albumin was eliminated by a Montage Albumin Deplete Kit (Millipore, Billerica, MA). Then, these were immediately stored at  $-80^{\circ}\text{C}$  until analysis.

### Lectin blotting

We applied the technique of lectin blotting (Savage et al., 1992) for detection of Con A binding glycoproteins. Each sample containing total protein of  $5\text{ }\mu\text{g}$  was mixed with a sample buffer to a final concentration of  $12\text{ mM}$  Tris-HCl (pH 6.8),  $25\%$  glycerol,  $2\%$  SDS,  $14.4\text{ mM}$  2-mercaptoethanol and  $0.1\%$  bromophenol blue, and boiled in a boiling bath for 5 min. The mixtures and molecular weight markers (GE Healthcare, Buckinghamshire, United Kingdom) were separated by  $5\%$  to  $20\%$  SDS-PAGE in a running buffer (pH 8.3) containing  $25\text{ mM}$  Tris,  $192\text{ mM}$  glycine and  $0.1\%$  SDS. SDS-PAGE was performed by  $20\text{ mA}/56\text{ cm}^2$  (area) and  $1\text{ mm}$  (thickness) for 90 min. Separated proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore) previously activated into a transfer buffer containing  $16\text{ mM}$  Tris,  $120\text{ mM}$  glycine and  $10\%$  methanol at  $100\text{ mA}$  for 1 h. The membrane was blocked in a Block Ace Solution (Dainippon Sumitomo Pharma, Osaka,

**Table 1. Characteristics of CSF samples**

Group	Gender (M/F)	Age*	Status
AD [15]	8/7	$73.8 \pm 5.2$	Probable
DLB [5]	3/2	$77.2 \pm 3.4$	Probable
Control [8]	3/5	$73.3 \pm 8.5$	No cognitive impairments and diseases of the NS

[ ], number of subjects.

\* Mean  $\pm$  SD.

AD, Alzheimer's disease; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; F, female; M, male; NS, nervous system.

**Table 2. Characteristics of serum samples**

Group	Gender (M/F)	Age*	Status
AD [20]	7/13	$79.2 \pm 6.8$	Probable
Control [20]	4/16	$71.6 \pm 12.0$	No cognitive impairments and diseases of the NS

[ ], number of subjects.

\* Mean  $\pm$  SD.

AD, Alzheimer's disease; F, female; M, male; NS, nervous system.

Japan) for 1 h at room temperature with shaking. The blocked membranes were then rinsed with  $0.1\%$  Tween-20 in phosphate-buffered saline, and each was washed 3 times for 5 min with shaking at room temperature. Then, the membranes were incubated with  $2\text{ }\mu\text{g/mL}$  Con A labeled biotin (J-Oil Mills, Tokyo, Japan) overnight at  $4^{\circ}\text{C}$ . Finally, biotinylated Con A binding glycoproteins were reacted with Streptavidin-Conjugated Alkaline Phosphatase (1:1000 dilution) (Promega, Madison, WI) for 2 h at  $25^{\circ}\text{C}$  and visualized with Western Blue (Promega). The Con A binding activities of each band were analyzed by Multi Gauge version 2.1 software (Fuji Film, Tokyo). The difference of staining of each membrane was compensated by the background intensity of area took from the original intensity of glycoprotein band.

### Coomassie brilliant blue staining

Every protein in the SDS-PAGE gel was stained with coomassie brilliant blue solution containing

50% methanol, 0.5 mg/mL coomassie brilliant blue and 10% acetic acid for 20 min at room temperature with shaking. Also, the gel had its color removed with 25% ethanol and 8% acetic acid at room temperature with shaking until positive protein bands appeared.

### **Silver staining**

The amount of CSF protein was lower than the serum protein, and the sensitivity of protein detection in coomassie brilliant blue staining was lower than that of silver staining. We could not visualize the protein of CSF in coomassie brilliant blue staining, and so we performed silver staining of the protein in the gel. The gel was swamped into 50% methanol for 10 min at room temperature with shaking then washed with distilled water for 10 min at room temperature. Sodium thiosulfate (0.02%) was used for intensifying the reaction for 3 min, followed by incubation with silver nitrate solution containing 1 mg/mL silver nitrate in 0.34% sodium hydroxide and 1.7% ammonia for 20 min at room temperature with shaking. Then, each gel was washed 4 times with distilled water for 10 min at room temperature, and developed with 2% sodium carbonate in 0.004% formaldehyde at room temperature with shaking. To stop the reaction, 7% acetic acid solution was used for 10 min at room temperature.

### **Statistical analysis and receiver operating characteristic analysis**

Intensities of Con A binding activities of glycoproteins were compared with Mann-Whitney's *U*-test: among the AD, the DLB and control groups for the CSF; and between the AD and control groups for the serum. We also compared the levels of CSF Con A binding glycoproteins among the 3 groups with analysis of variance. Con A binding glycoproteins which showed significant differences by statistical analysis in the CSF and serum were analyzed by receiver operating characteristic analysis for indicating sensitivity and specificity.

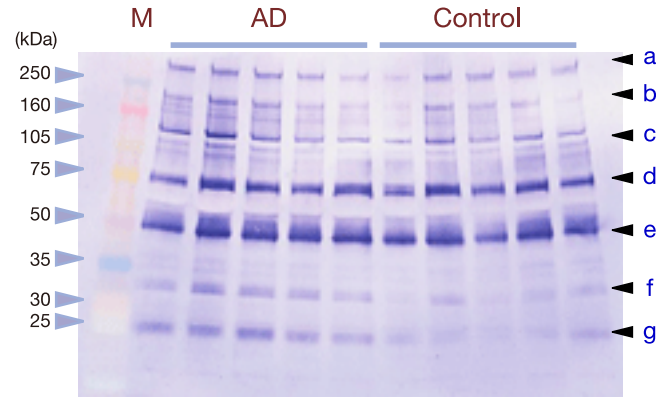
## **Results**

### **Lectin blotting**

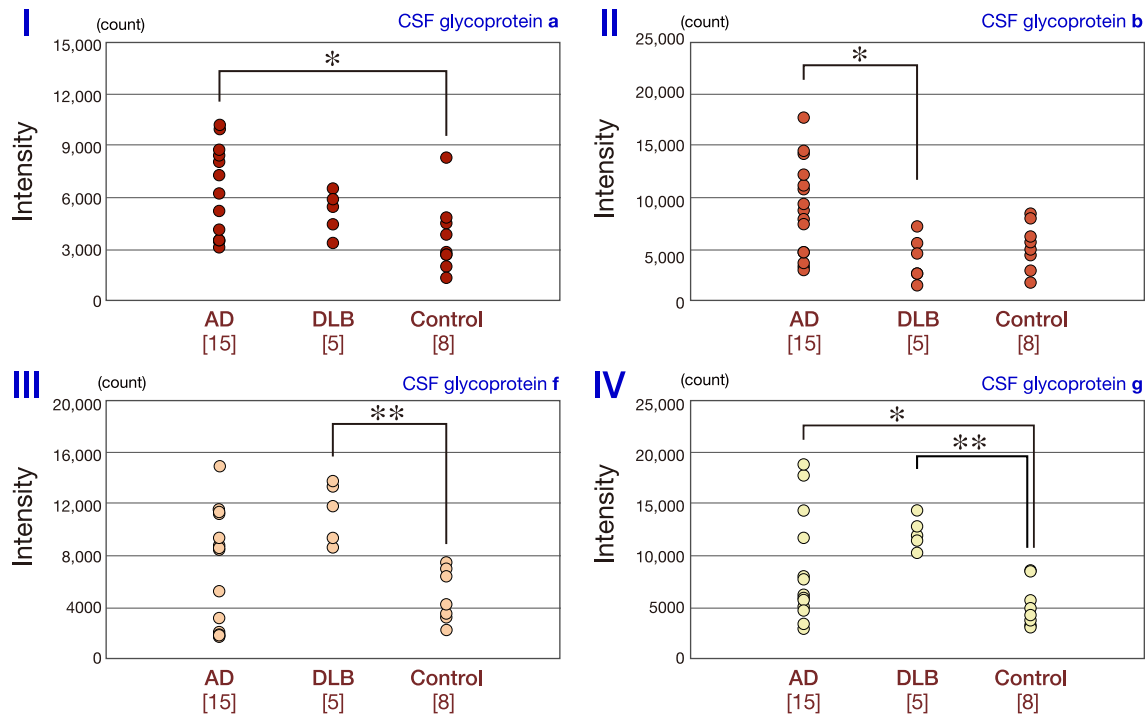
We detected some Con A binding glycoproteins in the CSF by lectin blotting, and tentatively named them **a** (over 250 kDa) to **g** (about 25 kDa) in descending order of molecular weight (Fig. 1). Con A binding activities of the 4 glycoproteins in the CSF are shown in Fig. 2.

Figures 2-I to -IV indicate intensities of Con A binding activities of CSF glycoproteins. The levels of Con A binding activities of glycoproteins **a** ( $6,477 \pm 2,520$ ) (mean  $\pm$  SD) and **g** ( $9,650 \pm 5,557$ ) from the CSF in the AD group were significantly higher than those of **a** ( $3,978 \pm 2,133$ ) and **g** ( $4,779 \pm 1,746$ ) in the control group ( $P < 0.05$ ). Con A binding glycoprotein **a** had 88.89% sensitivity and 66.67% specificity (cut off 5026) and **g** had 77.78% sensitivity and 73.77% specificity (cut off 5783) between the AD group and the control group. Additionally, the Con A binding activity of glycoprotein **b** (between 250 and 160 kDa) in the AD group was also higher than that of the DLB group ( $P < 0.05$ ) and that had 100.0% sensitivity and 66.67% specificity (cut off 7303) between those 2 groups, but there was no significant difference between the AD and control groups. The intensity of **f** (between 35 and 30 kDa) and **g** in the DLB group were significantly higher than in the control group ( $P < 0.01$ ) and those Con A binding glycoproteins had 100.0% sensitivity and 100.0% specificity (cut off 8010: **f**, 9503: **g**). The intensities of CSF Con A binding glycoprotein **b**, **f** and **g** had significant difference among 3 groups ( $P < 0.05$ , analysis of variance).

We performed lectin blotting of Con A binding glycoproteins in the serum, and called **A** (between 250 and 160 kDa) to **J** (about 25 kDa) in descending order of molecular weight (Fig. 3). Figure 4 shows intensities of Con A binding activities of glycoproteins in the serum. In the AD group, the Con A binding intensity of glycoprotein **H** (between 50 and 35 kDa) ( $6,234 \pm 2,692$ ),



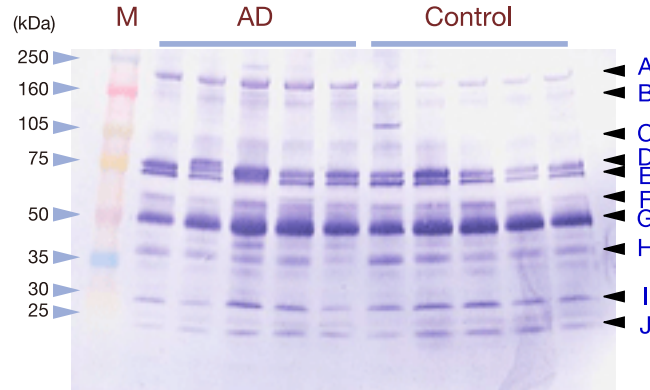
**Fig. 1.** Banding patterns of concanavalin A (Con A) reactive glycoproteins in the cerebrospinal fluid (CSF) detected by lectin blotting with Con A. Glycoproteins analyzed in this study (black arrow heads) are tentatively named **a** to **g**. Lane M: molecular markers; AD, Alzheimer's disease patients; control, non-dementia control subjects.



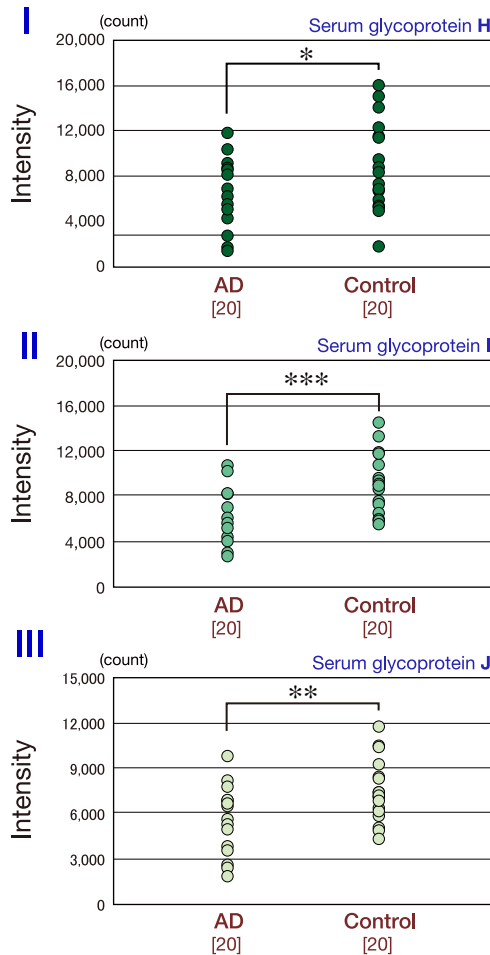
**Fig. 2.** Concanavalin A (Con A) binding activities of the cerebrospinal fluid (CSF) glycoprotein **a**, **b**, **f** and **g** determined by densitometry are seen in 2-I, -II, -III and IV, respectively. Shown are intensities of each samples. \* $P < 0.05$ , \*\* $P < 0.01$ : comparison between the Alzheimer's disease (AD) group and the dementia with Lewy bodies (DLB) group or control group, or both (Mann-Whitney's  $U$ -test). [ ], number of subjects.

**I** (about 30 kDa) ( $5,765 \pm 2,290$ ) and **J** (about 25 kDa) ( $5,291 \pm 2,089$ ) were significantly lower than the control group:  $8,851 \pm 3,470$  ( $P < 0.05$ ),  $8,817 \pm 2,581$  ( $P < 0.001$ ),  $7,564 \pm 2,235$  ( $P < 0.01$ ), respectively. Additionally, Con A binding glycoproteins **H**, **I** and **J** had 70.00% sensitivity and

60.00% specificity (cut off 6454), 80.00% sensitivity and 75.00% specificity (cut off 6311) and 85.00% sensitivity and 65.00% specificity (cut off 5657). Other Con A binding glycoproteins had no significant differences between AD and other groups (data not shown).



**Fig. 3.** Banding patterns of concanavalin A (Con A) reactive glycoproteins in the serum detected by lectin blotting with Con A. Glycoproteins analyzed in this study (black arrow heads) are tentatively named **A** to **J**. Lane M: molecular markers; AD, Alzheimer's disease patients; control, non-dementia control subjects.



**Fig. 4.** Concanavalin A (Con A) binding activities of serum glycoproteins **H**, **I** and **J** determined by densitometry are seen in I, II and III respectively. Shown are intensities of each samples. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ : comparison between the Alzheimer's disease (AD) group and control group (Mann-Whitney's  $U$ -test). [ ], number of subjects.

### Semiquantification of glycoproteins in the CSF and serum

We also analyzed protein levels of CSF and serum glycoproteins which showed significant intergroup differences with lectin blotting. As proteins can be stained directly with coomassie brilliant blue or silver staining, we could compare the amount of glycoprotein. Differences in protein levels of CSF Con A binding glycoproteins **a**, **b**, **f** and **g** were not significant between groups which had significant differences in Con A binding activities. Differences in the amount of coomassie brilliant blue-stained serum glycoproteins **H**, **I** and **J** were not significant between the AD and control groups.

### Discussion

In AD patients, some Con A binding glycoproteins were altered in the CSF and serum. These data indicate that the CSF Con A binding glycoproteins in **a** and **g** show the separation between the AD group and the control group. However, in both CSF and serum, glycoproteins with different Con A binding activities were not distinguished by the amount. From this we figured that the al-

teration was due to a sugar chain rather than to the amount of glycoprotein. Additionally, the alteration of the sugar chain specifically occurring at the mannose site was the reason for using Con A lectin in this study. Recently, there have been some reports which suggest the relationship between glycosylation and AD. An unusual glycoform of acetylcholinesterase, which does not bind to Con A, is increased in the AD brain and CSF (Sáez-Valero et al., 1999, 2000). Wheat germ agglutinin-binding glycoproteins are decreased in the CSF of AD patients (Fodero et al., 2001), and there is a relationship between aberrant glycosylation and the pathology of AD (Ahmed and Thornalley, 2002), while several glycoproteins are altered in AD (Puchades et al., 2003). These results support our findings and suggest that a sugar chain of glycoproteins is altered in AD. Moreover, it was possible to distinguish glycoproteins **f** and **g** in the DLB group from the control group. The glycoprotein **b** in the CSF might be separated the AD group from the DLB group. The CSF glycoproteins **b**, **f** and **g** indicated aberrant glycosylation also occur in DLB, and this fact suggests that alteration of mannose glycosylation of Con A binding glycoprotein might be a new biomarker in not only AD but also other dementia such as DLB.

On the other hand, we found that there was no significant difference in CSF glycoprotein **d** using lectin blotting with Con A in this study, although wheat germ agglutinin-binding activity was significantly lower than in the AD group in previous study (data not shown). Wheat germ agglutinin specifically binds to  $\alpha$ -N-acetylglucosamine and terminal sialic acid (Nagata and Burger, 1974; Monsigny et al., 1980). These results suggested aberrances in glycosylation at  $\alpha$ -N-acetylglucosamine and/or sialic acid sites, but not at the mannose site. In this way, using some lectins in the same glycoprotein might be able to clarify the site of altered sugar chain.

In AD, aberrant glycosylation of glycoproteins was found ubiquitously in the CSF, but those found in the serum were limited to the glycoproteins with relatively smaller molecular weights.

Various molecular weights of Con A binding glycoproteins from the CSF such as **a**, **b**, **f** and **g** were found to have the potential of an aberrant sugar chain. The change was found in Con A binding glycoproteins with both large and small molecular weights in the AD group and/or DLB group but not in the control group in the CSF, which is probably caused by a variation in glycosylation. However, altered Con A binding activities of serum glycoproteins were observed in glycoproteins with less than 40 kDa. In the serum of AD patients, aberrant glycosylation suggested by Con A binding activity was limited to the glycoproteins with smaller molecular weights.

The central nervous system is isolated from the blood circulation by the blood-brain barrier and blood-cerebrospinal fluid barrier because of its physiological importance. These barriers have been thought to protect the brain from various harmful materials in the circulation as static walls (Segal, 2000; Begley and Brightman, 2003; Nitta et al., 2003). In addition, membrane proteins promote cell membrane permeability of a low molecular weight compound, called a transporter, and these membrane proteins have been thought to relate to the active efflux of harmful materials from the brain (Kusuhara and Sugiyama, 2001; Lee et al., 2001; Sun et al., 2003; Begley, 2004). Therefore, we considered that aberrant glycosylation of glycoproteins with a relatively large molecular weight which was directly or secondarily generated could not be excreted into the circulating blood because of the blood-brain barrier and/or blood-cerebrospinal fluid barrier: these glycoproteins might have been excreted into other metabolic pathways instead of those. On the other hand, relatively smaller glycoproteins could be passed through the blood-brain barrier and/or blood-cerebrospinal fluid barrier into the circulating blood. Consequently, a metabolic pathway using blood flow might exist for those glycoproteins as one possible pathway.

In the future, it will be necessary to identify the glycoproteins altered in AD. However, we found that glycosylation of Con A binding gly-



coproteins in the AD group was different in both CSF and serum. Alteration of Con A binding activities might especially have some use as a new biomarker in the serum. On the other hand, this study has some difficult issues such as increasing of respective samples, identification of molecular weight of each Con A binding glycoprotein and investigation of DLB or other non-AD dementia in the serum. In the immediate future, those problems will be solved and clarifying the mechanism of variant glycosylation in AD might contribute not only to the discovery of a new biomarker but also to the resolution of a new pathology.

**Acknowledgments:** We thank Dr. Kenji Wada, Dr. Yosuke Wakutani and Dr. Kenji Nakashima of the Department of Neurology, Institute of Neurological Sciences, Tottori University Faculty of Medicine for providing us the CSF and serum samples. We also would like to thank Mr. Tim Wiltshire, Tottori University Faculty of Medicine for checking the English of this article.

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*Received July 25, 2007; accepted January 4, 2008*

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